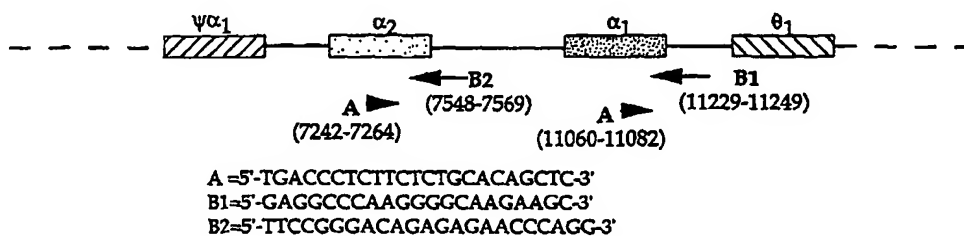




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(54) Title: A RAPID METHOD FOR DIAGNOSING THE VARIOUS FORMS OF α -THALASSEMIA

**(57) Abstract**

The present invention relates to the simultaneous and specific identification of the variant forms of α -thalassemia. This invention utilizes simple and readily available equipment to rapidly identify, diagnose and differentiate the different forms of α -thalassemia. A simple and rapid non-radioisotopic technique for the diagnosis and differentiation of the common forms of α -thalassemia has been developed. This approach works on any biological tissue including blood, wherein the assay works equally well with fresh blood and dried blood samples stored on filter paper.

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A RAPID METHOD FOR DIAGNOSING
THE VARIOUS FORMS OF α -THALASSEMIA

FIELD OF THE INVENTION

The present invention relates to the field of diagnostic methodologies. More particularly, the invention is related to a simple, inexpensive and rapid method for detecting thalassemias and monitoring therapeutic effects on the disease.

BACKGROUND OF THE INVENTION

The α -thalassemia (" α -thal") are common genetic disorders that result from reduced synthesis of the α globin chains of fetal ($\alpha_2\gamma_2$, HbF) and adult ($\alpha_2\beta_2$, HbA) hemoglobin (Weatherall and Clegg, 1981; Higgs et al., 1989, Higgs and Weatherall, 1993). The normal human α globin gene cluster is located on the short arm of chromosome 16 (Breuning et al., 1987; Buckle et al., 1988). In α -thal syndromes, α -globin synthesis is either diminished or absent due to either deletional or non-deletional abnormalities involving the α -globin genes (Higgs et al., 1989; Higgs and Weatherall, 1993). Diploid cells have four α -chain genes (i.e., $\alpha\alpha/\alpha\alpha$). The severity of the hematological and clinical picture is directly proportional to the number of involved α -globin genes and thus the deletion of one, two, three, or all four of these α genes attribute to mild to complete α chain deficiencies syndromes.

The deletion of α -chain at birth results in the formation of a γ chain tetramer, Hb Bart's (γ_4). The percentage of Hb Bart's present corresponds to the degree of α chain deficiency (Cong & Shong, 1982; Liang et al., 1985; Higgs et al., 1989; Higgs and Weatherall, 1993). The α -thal-2 genotype has been found to have one of the two genes deleted, thus these heterozygotes ($\alpha\alpha/-\alpha$) possess a mild α chain deficiency resulting from the presence of only three α chain genes and have not more

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° than 2% Hb Bart's at birth (Higgs et al., 1989).
Homozygotes ($-\alpha/-\alpha$), as well as α -thal-1 heterozygotes ($\alpha\alpha/--$), possess moderate α chain deficiency resulting from the presence of only two α chain genes and have approximately 5% Hb Bart's at birth (Higgs and Weatherall, 1993). While Hb H disease is a heterozygosity for α -thal-2 in association with an α -thal-1 heterozygosity ($--/-\alpha$), a severe α chain deficiency occurs due to the deletion of three α chain genes (Higgs et al., 1989; Higgs and Weatherall, 1993). The α -thal-1 homozygotes ($--/--$), which lack any functional α genes, present with Hb Bart's is known as hydrops fetalis syndrome and results in intra-uterine or early post-delivery death to the fetus (Lie-Injo & Hie, 1960; Higgs et al., 1989). Mother bearing fetuses with homozygous α -thal-1 ($--/--$) are at high risk for obstetrical complications, such pregnancy induced hypertension, eclampsia, and/or death.

At present, for a variety of technical, logistical and economical reasons, large-scale carrier screening and appropriate ante-natal detecting programs have not been established for the populations in which this syndrome occurs (Bowden et al., 1992; Higgs and Weatherall, 1993).

It is an object of the invention to develop a diagnostic method and kit for the detection and quantitation of hemoglobin (Hb) α gene(s) in α -thalassemia patients.

It is another object of the present invention to develop a method and kit for screening for carriers of the genetic disorder, α thalassemia.

It is yet another object of the present invention to identify persons who are at risk of having offspring with homozygous α -thalassemia as well as identify α -thalassemia in individuals with unexplained microcytosis and hypochromia.

Another object of the present invention is to develop a sensitive, non-radioisotopic test capable of differentiating between the various forms of α -

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- ° thalassemia, by detecting and quantitating α gene(s) from blood samples.

SUMMARY OF THE INVENTION

The present invention relates to the identification
5 of primers capable of detecting and distinguishing between
the various forms of α -thalassemia. The α -globin primers
of the present invention are derived from the α -globin
genes. One such primer comprises a region common to both
 α_1 and α_2 genes. A second such primer is specific for α_2
10 gene and a third primer is specific for α_1 gene. These
primers are designed to specifically hybridize to portions
of the hemoglobin α genes, such that, when amplified with
an inducing agent, give identifiable amplification
products. The presence and amount of the products is
15 correlated to specific forms of α -thalassemia, based upon
the type of gene deletion associated with the disease.

The present invention relates to a method for
identifying and differentiating among the various forms of
 α -thalassemia. The method of the present invention uses
20 highly specific primers to identify and differentiate
among the forms of α -thalassemia. The assay may include a
nucleic acid amplification step wherein the hemoglobin α
genes are simultaneously amplified and detected. In
another embodiment of the present invention, a color
25 complementation assay is used to provide a convenient,
rapid, non-radioisotopic assay system for quantitation of
the α gene(s).

A kit comprising at least three primers, capable of
detecting, quantitating and distinguishing α -thalassemia
30 variants is also encompassed in the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Old world distribution and prevalence
of α -thalassemia.

35 Figure 2. Schematic representation of primers
design for detecting the deletion of hemoglobin α genes.

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Figure 3. Coamplification of $\alpha 1$ - and $\alpha 2$ - globin genes from blood samples of normal and α -thal syndrome patients. Three of each category of tested subjects are shown.

Figure 4. Gel scanning data from both normal and different genotypes of α -thal syndrome patients.

Figure 5. PCR-mediated CCA from normal and two α -thal syndrome patients. Panel A shows the amplified α genes with different colors in the reaction tubes as viewed with UV-light. In lane 1 is an orange color, the result of an equal mixture of red and green fluorescein dye product amplification; lane 2 displays green due to the absence of red ($\alpha 2$) amplification; lane 3 is colorless due to the total absence of alpha genes (---/---) for amplification. Panel B: the amplified α genes were separated by electrophoresis on a TBE gel and visualized under UV-light by the color and size of each α gene fragment.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the identification of nucleic acid primers capable of detecting and distinguishing between the various forms of α -thalassemia.

The primers of the present invention are used to identify and differentiate between the various forms of α -thalassemia. Samples to be tested using the present invention include blood, either dried or whole blood. In addition, samples may be derived from any biological tissue containing nucleic acid material, including but not limited to skin tissue, amniotic fluids, umbilical cord, dried tissue and paraffin embedded tissue.

A particular advantage of the present invention is its adaptability to samples in various forms; that is, the present invention can detect the various forms of α -thalassemia from fluid or dry biological samples.

The term "internal standard DNA" means a nucleic acid which will be present in a sample whether or not the

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target DNA is present and which can be labeled and detected independently of the globin target genes. For example, the internal standard DNA may correspond to a known sequence of DNA, i.e. a gene, on a chromosome different from that on the globin target genes.

5 The term "oligonucleotide" as used herein in referring to primers, probes, oligomer fragments to be detected, oligomer controls and unlabeled blocking oligomers is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably
10 more than three. Its exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide.

 The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a
15 purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, i.e. in the presence of
20 nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH. The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to
25 separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The exact lengths of the
30 primers will depend on many factors, including temperature, source of primer and use of the method. For example, for diagnostics applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides,
35 although it may contain fewer nucleotides. For other applications, the oligonucleotide primer is typically

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shorter, e.g., 7-15 nucleotides.

The primers of the present invention are selected from regions of the genomic sequence of the hemoglobin α genes. The 5' primer is preferably located at the 5' end of each of α_1 and α_2 gene and serve as a common sense primer. Most preferred is a 5' primer comprising the sequence 5'-TGACCCTCTTCTCTGCACAGCTC-3' (SEQ ID NO:1) corresponding to a common region in both the α_1 and the α_2 genes. This sequence, referred to herein as "A primer," is found at position 7242-7264 in the α_2 gene and at position 11060-11082 in the α_1 gene of the genomic sequence described by as set forth in Gen Bank, Accession No. J00153, also known as HUMHBA4.

Two different 3' primers provide the specificity to detect multiple α gene deletions. These primers are preferably 3' antisense sequences of the hemoglobin α genes. A preferred α_2 3' primer comprises a region within the 3' untranslated portion of the α_2 gene. One such primer comprises 5'-TTCCGGGACAGAGAGAACCCAGG-3' (SEQ ID NO:3). This sequence, referred to herein as "B2 primer," is found at position 7512-7534 in the genomic sequence as set forth in Gen Bank, Accession No. HUMHBA4. A second preferred α_1 3' primer comprises the sequence 5'-GAGGCCCAAGGGGCAAGAAGC-3' (SEQ ID NO:2) corresponding to a region within the 3' untranslated portion of the α_1 gene. This sequence, referred to herein as "B1 primer," is found at position 11229-11249 in the genomic sequence as set forth in Gen Bank, Accession No. HUMHBA4.

The primers of the present invention can be used in various ratios in detection of the different forms of α -thalassemia. Ratios selected will depend upon several factors, including the method of detection used, the amount of nucleic acid material and the source of the biological sample. When using amplification techniques, it is preferred that the 5' common sense primer is present

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at a ratio range of 1-2 and the 3' α_2 and 3' α_1 primers are present at a ratio range of 0.1-1.5. A particularly preferred embodiment when using amplification techniques is 5' common sense primer: 3' α_1 primer, 3' α_2 primer ratio of about 1:1:1.

5 The primers may vary in size but preferably contain a substantial portion of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:3. Specific nucleotide positions of the primers may be varied so long as the primers retain then ability to specifically hybridize to the hemoglobin α genes.

10 Primers are readily synthesized by standard techniques. Detailed procedures for the phospho-triester and hydrogen phosphonate methods of oligonucleotide synthesis are described in the following references, which are incorporated by reference: Caruthers et al., US Patent
15 Nos. 4,458,066 and 4,500,707; Matteucci et al., *J. Amer. Chem. Soc.* 103:3185-3191 (1981); Caruthers et al., *Genet. Engin.* 4:1-17 (1987); Jones, Chapter 2 and Atkinson et al., Chapter 3, in Gait, ed., *Oligonucleotide Synthesis: A practical Approach* (IRL Press, Washington, DC, (1984);
20 Froehler, et al., *Tetrahed. Lett.* 27:469-472 (1986); Garegg, et al., *Tetrahed. Lett.* 27:4051-4058 (1986); and Froehler, et al., *Nucl. Acid Res.* 14:5399-5407 (1986).

The primers of the present invention can be used for amplification using standard polymerase chain reaction
25 techniques. Mullis US Patent Nos. 4,683,202 and 4,683,195 describe the basic amplification technique and are incorporated herein by reference. Other variant amplification techniques can also be used. Such techniques are known to the skilled artisan.

30 Once amplified, the products can be analyzed by many techniques known in the art. For example, one technique uses physical separation of the amplification products to distinguish the products. Physical separation can be carried out by various methods, including but not limited
35 to filtration, electrophoresis and chromatography techniques.

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0 Detection and differentiation of the allele-specific
amplification products may also be accomplished using
immunocytochemistry methods relying on reagents that
associate with one another with high specificity and
affinity such as biotin-streptavidin, digoxigenin and
5 others (Didenko, et al., 1996).

A further detection method would allow for high DNA
through-put in the context of mass screening employs
detection of alpha-globin gene(s) on the surface of a
charge coupled device, currently known as a "DNA-chip"
10 (Lamture, et al., 1994).

A preferred method of detecting the presence and
amount of each of the amplified products is one that
allows for the detection of more than one target DNA
without the need to separate the different amplified
15 products. For example, the primers of the present
invention may be used in a color complementation assay, as
described in US Patent No. 5,489,507, incorporated herein
by reference. In brief, this embodiment comprises the
steps of (i) simultaneously amplifying the target
20 hemoglobin genes and one or more internal standard DNAs in
a sample, (ii) providing one or more first labeling means
capable of binding to the target hemoglobin genes in the
sample, (iii) providing one or more second labeling means
capable of binding to one or more internal standard DNAs
25 in the sample, (iv) combining the first labeling means and
the second labeling means with the sample so that the
first labeling means binds to their target hemoglobin
genes to form one or more labeled target hemoglobin genes
and the one or more second labeling means bind to their
30 respective internal standard DNAs to form one or more
labeled internal standard DNAs, (v) separating the unbound
first labeling means from the sample, and (vi)
illuminating the sample with an illumination beam having a
predetermined wavelength characteristic such that a
35 distinct color signal is produced whose character depends
on which of the one or more target DNAs that have been

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° amplified. Preferably, the steps of simultaneously
amplifying, providing a first labeling means, providing
one or more second labeling means, and combining are
achieved by carrying out a polymerase chain reaction
wherein the first labeling means comprises a pair of
5 primers specific for the target globin genes, at least one
of the primers being labeled directly or indirectly with a
first color-producing or color-absorbing label, and
wherein the one or more second labeling means comprise
separate pairs of primers, at least one member of each
10 pair being labeled directly or indirectly with a different
second color-producing or color-absorbing label.

Color-producing labels and color-absorbing labels can
be attached to primers in a variety of ways either
directly or indirectly. Mathews et al (1988) provide a
15 comprehensive list of both direct and indirect labeling
means for nucleic acids. Accordingly, this reference is
incorporated by reference.

Labels can be directly attached to primers via a 5'
amino or thiol linking group, e.g. Connolly et al (1985)
20 and Fung et al., US Pat. No. 4,757,141. Many commercially
available dyes with amino- or thiol-reactive moieties can
be used as color-producing and/or color-absorbing labels.
For example, the following dyes suitable for use with the
invention are available: (i) thiol-reactive; 5-
25 iodoacetaminofluorescein, fluorescein-5-maleimide,
tetramethylrhodamine-5(and-6) iodoacetamide, rhodamine X
iodoacetamide, and the like, and (ii) amino-reactive;
Fluorescein-5 (and-6) isothiocyanate, Texas Red, rhodamine
X isothiocyanate, fluorescein-5(and-6)
30 succinimidylcarboxylates, 7-amino-4-methylcoumarin-3-
acetic acid, 5- and/or 6-succinimidylcarboxylates of
rhodamine dyes, and the like, and dyes disclosed in US
Pat. Appln Serial no. 07/138,287 filed 24-Dec-1987.

Color-producing and/or color-absorbing labels can
35 also be attached to primers indirectly via antibodies or
via biotin and avidin or streptavidin. Means for

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° synthesizing biotinylated primers is well known in the art (e.g. Chollet et al. 1985; Agrawal et al., 1986). Biotinylating reagents are also commercially available and hence are known and available in the art.

5 One or more different internal standard DNAs can be amplified with primers having the same label in order to vary the relative concentrations of the color-producing or color-absorbing labels at the time of analysis. For example, if one of the color producing labels is a fluorescent dye with low fluorescent efficiency and a
10 given degree of brightness is required for color complementation, a number of internal standard DNAs can be labeled with the same "weak" fluorescent label to increase the labels relative concentration, and hence, its relative brightness.

15 Preferably, in automated embodiments of the present invention the first and second color-producing or color-absorbing means comprise pairs of amplification primers wherein the 5' nucleotide of one primer of the pair is covalently attached to biotin and wherein the 5'
20 nucleotide of the other primer is directly labeled with a color-producing or color-absorbing label. Preferably, color-producing labels are fluorescent labels, such as fluorescein, Texas Red, tetramethylrhodamine, dichlorodimethoxyfluoroscein, or the like. In some cases,
25 fluorescent labels can also act as color-absorbing labels. Preferably, color-absorbing labels are used with a first labeling means and are organic indicator molecules which can be linked to an amino- or thiol-derivatized primer and whose absorption spectrum substantially overlaps both the
30 emission spectrum of the illumination beam and labeling means. That is, preferably, the color-absorbing label is associated with the target DNA and the fluorescent label used in conjunction with a second labeling means. That is, preferably, the color-absorbing label is associated with
35 the target DNA and the fluorescent label is associated with an internal standard DNA. If a target DNA is present

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° and amplified the measured absorption will be high and fluorescence will be low. The reverse holds if the target DNA is not present. The presence of the target DNA is determined by the ratio of absorption to fluorescence intensity. Most preferably, fluorescent labels such as
5 those disclosed in Khanna et al., US Pat. No. 4,318,846; 4,439,356; and 4,481,136.

As mentioned above, for automated embodiments it is preferred that the first and second labeling means comprise pairs of primers, one with biotin attached to its
10 5' end and the other with a color-producing or color-absorbing label attached to its 5' end. In such an embodiment, after amplification, the reaction mixture containing the amplified chains are exposed to an avidin or streptavidin derivatized substrate, e.g. magnetic
15 microspheres (Advanced Magnetics, Inc., Cambridge, Mass.). This allows for rapid separation of the amplified chains from the rest of the reaction mixture which, for example, may contain a preponderance of labeled primers associated with the target DNA if the target DNA is not present in
20 the sample (and consequently not amplified). Separation is readily carried out by filtration, or if magnetic microspheres are used, by magnetic separation. The labeled strands of the amplified DNA are used, by magnetic separation. The labeled strands of the amplified DNA are
25 separated from the biotinylated strands (and primers) by denaturation procedures, e.g. exposure to strong alkali solution (e.g. between 0.1 and 1.0 procedures, e.g. exposure to strong alkali solution (e.g. between 0.1 and 1.0 NaOH), or heat plus exposure to a solution of
30 formamide and water. The biotinylated DNA is then removed with the substrate, and the remaining solution containing the labeled strands is illuminated by the illumination beam. Automation of the invention is readily carried out by use of a general purpose laboratory robot, such as the
35 disclosed by Wilson et al., BioTechniques, Vol. 6, pgs., 776 (1988).

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All articles and patents referred to herein are incorporated, *in toto*, by reference.

While the invention is described above in relation to certain specific embodiments, it will be understood that many variations are possible, and that alternative materials and reagents can be used without departing from the invention. In some cases such variations and substitutions may require some experimentation, but will only involve routine testing.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention and others can, by applying current knowledge, readily modify and/or adopt for various applications such specific embodiments without departing from the generic concept, and therefore such adaptations and modifications are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments.

Example 1: Direct analysis of five most frequent α -thal syndrome genotypes ($-\alpha/\alpha$, $--/\alpha$, $-\alpha/-\alpha$, $--/-\alpha$ and $--/--$) by PCR amplification of DNA from 62 patients' blood samples.

Direct analysis of 5 most frequent α -thal genotypes by PCR amplification of DNA samples from α -thal patients.

1). Primer designing.

To examine the presence of different types of α gene(s) deletion, three primers were designed for specifically coamplifying α_1 and α_2 genes from single blood sample by PCR. Primer B₁ and B₂ are respectively 3' antisense primer of α gene. Primer A, located within the 5' portion of each α_1 and α_2 gene, has an identical DNA sequence for both α_1 and α_2 gene and serve as a common sense primer. The primers for β -actin gene (sense primer 1854 bp-1874 bp) used as an internal control. Primer design for α genes is illustrated in Figure 2. After PCR three amplified DNA fragments: α_2 gene (292 bp), α_1 gene (190 bp), and β -actin gene (105 bp) could be observed from 2% NuSieve gel stained with ethidium bromide.

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2). Optimal condition for PCR amplification.

Genomic DNA (0.3 to 1 ug) is mixed with 5 μ l reaction buffer, 4 μ l of 125 μ M dNTP, and 1 μ l of each 25 μ M primer (primers A, B₁ and B₂ for α genes, as well as sense and antisense primers for β -actin). The reaction is performed in 50 μ l volume. The mixture is heated at 95°C for 5 minutes to denature the DNA before 1.25 units Taq polymerase is added into the solution. Thirty cycles are performed on a Perkin-Elmer Cetus DNA thermal Cycler: denaturing at 94°C for 1.0 min., annealing at 60°C for 1.0 min., and extending at 72°C for 1.0 min. After PCR reaction, 10-15 μ l of the amplified product are analyzed on 2% NuSieve gel by electrophoresis. Three bands, corresponding to 190 bp for α_1 gene, 292 bp for α_2 gene and 105 bp for β -actin gene, were visualized. By comparing the intensity of each α_1 or α_2 gene bands on ethidium bromide-stained gel between normal and α -thal patient, the deletion status of α genes could be accessed.

3). Detection of α gene deletion from α -thal syndrome patients.

Genomic DNA was isolated from either whole blood or dried blood samples embedded on a filter paper from 21 normal subjects and 62 α -thalassemia patients. This DNA was used as a template to amplify by PCR three DNA fragments (α_2 , α_1 and β -acting genes) which were easily identified from each sample tested. In normal 4 genes ($\alpha\alpha/\alpha\alpha$) samples, all three bands showed almost equal intensity, which indicated α_1 or α_2 genes are present normally without any gene deletion (Figure 3, lanes 1-3). When the intensity of each amplified α gene was compared with the intensity of the β -actin gene band (a gene product which serves as an internal control), deletion of α_1 or α_2 gene could be identified from ethidium bromide-stained NuSieve agarose gel. Such heterozygotes ($-\alpha/\alpha\alpha$) possess a mild α -chain deletion resulting from the presence of only three α genes. The band for α_2 gene (292 bp) was found to have less intensity (half intensity of

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normal α_2 gene band) as compared to α_1 gene (190 bp) Figure 3, lanes 4-6). α thal-1 Heterozygotes ($--/\alpha\alpha$) as well as homozygotes ($-\alpha/-\alpha$), possessed moderate α -chain deficiency resulting from the presence of only two α -chain genes, in which less intensity of both 190 bp and 292 bp bands (Figure 2, lanes 10-12) were observed. However Hb H disease ($--/-\alpha$) resulted from deletion three of α -gene showed that complete loss of 292 bp band and less intensity of α_1 band compared with β -actin band (Figure 3, lanes 13-15), while the Hb Bart's hydrops fetalis syndrome ($--/--$) had neither α_2 nor α_1 gene band (Figure 3, lanes 16-18). Using this set of PCR primers, different band intensity from each target gene could reflect the genotype status of α gene detection in α -thal patients. A total of 21 normal samples and 62 α -thal patients representing different α gene deletions were examined by this assay.

Example 2. Quantitation of PCR-amplified hemoglobin alpha gene using a photo-densitometer with statistical estimations of band intensities.

In order to obtain quantitative values from this assay, a negative film taken from ethidium bromide stained gel was applied to a densitometer (UltrascanXL enhancer laser densitometer, LKB) to quantitate the area under curve reflecting the intensity of each band. As shown in Figure 4, different α -gene deletions exhibited different gel scanning patterns of the three peaks (corresponding to β -actin, α_1 and α_2 band). The pattern of the peaks correlated very well with the status of α gene deletion, and each genotype has its own characteristic pattern of the peaks. For example, in ($-\alpha/\alpha\alpha$) and α_2 peak is much lower than that of α_1 band, while the α_1 peak is obviously much higher than that of the β -actin [Figure 4(2)]; In ($--/\alpha\alpha$), both α_1 and α_2 peak are lower than that of β -actin [Figure 4 (3)]; In ($-\alpha/-\alpha$), the α_2 peak diminished, only peaks for α_1 and β -actin were observed [Figure 4 (4)].

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The intensity of each band could be represented by the area under the curve. The copy number of a gene then is determined by either the ratio of the area for α gene band to that of β -actin gene, or by comparing the percentage of area from each peak. The average values of ratio and the percentage of peak area are summarized in the Table 1. There are significant differences between each category of the α -thal syndromes.

The ratios of band intensity among α_1 over β -actin or α_2 over β -actin in normal subject was 1.10 ± 0.25 and 0.70 ± 0.35 , respectively, while in α -thal patients with different genotypes a significant change in the calculated values were seen (Table 1). The two clinical significant types of α -thal syndrome, $(-\alpha/\alpha\alpha)$ and $(--/\alpha\alpha)$ could be identified either comparing the percentage of each band or ratios of α_1/β -actin and α_2/β -actin.

Example 3. **Detection of hemoglobin α gene by using DNA-PCR-mediated color complementary assay (CCA) from both normal and α -thal patients.**

1) Experimental design

Another quicker (without gel electrophoresis), PCR-mediated color complementary assay (CCA) for detecting α globin gene(s) deletion was developed. It is based on the simultaneous amplification of two α genes with fluorescent-labeled oligonucleotide primers and the visualization of the amplified-color gene products by UV light irradiation (Chehab and Kan, 1989). Thus, the generation of a color or combination of colors from the patient samples can be visualized under UV light in the tube without electrophoresis. In this CCA, 2 different fluorophores were coupled to B1 or B2 primer in order to detect and differentiate globin gene α_1 or α_2 . In the same PCR mixture B1 or B2 primer that are differently labeled with either fluorescein or rhodamine were added. The primer B1 was labeled with 5'-carbon fluorescein (FAM) appears with a green color, while the B2 primer was

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labeled with 6-carboxyl-X-rhodamine (ROX) showing red color. The common 5' primer was unlabeled primer A. After coamplification of both α_1 and α_2 colored-PCR products were produced in the reaction tubes.

2) Optimal condition for PCR-CCA.

The oligonucleotide B1 and B2 with a primary amino group attached to the 5' end were synthesized. The dye-labeled oligonucleotide were then purified from the nonconjugated oligonucleotide by HPLC Aquepore 300C-8 column. The recovered fluorescent oligonucleotide were then dried down and resuspended in sterile distilled water. Measurement of the absorbance of the oligos at 260 nm was performed and diluted to a final concentration of 25 μ M. Aliquot of the dye-labeled primers are stored at -20°C in the dark until use.

PCR mediated CCA reaction was processed as follows: 2 μ l of each primer A, FAM-B1 and ROX-B2 was mixed with 1 μ g of genomic DNA in a 50 μ l reaction mixture containing 5 μ l reaction buffer (50 mM KCl, 10mM Tris (pH8.0), 1.5mM $MgCl_2$), and 4 μ l each dATP, dCTP, dGTP, dTTP at 125 μ M. 5 units of native Taq DNA polymerase was added, and the reaction mixture was overlaid with 50 μ l of mineral oil. First, amplification reaction is performed at 94°C for 5 min., 60°C for 1 min., and 72°C for 1.0 min. Thirty amplification cycles were followed at 94°C for 1 min., 60°C for 1 min., 72°C for 1 min. Aliquots (10-15 μ l) from each amplification mixture were loaded on 6% TBE gel for electrophoresis to confirm the existence and size of amplified product. Also detected were PCR-CCA products by color reactions without electrophoresis. After PCR, amplified samples were separated from free primers by repeated ultrafiltration through Centricon 100 micro concentrators (Amicon). The tubes then were visualized on a long wavelength (300 nm) UV transilluminator and photographed with a Polaroid MP-4 Land Camera. Each film is exposed at f4.5 for 30 sec to 1 min. with a combination

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of Wratten gelatin filter no.16 and no.21. On the basis of the PCR-CCA data from the large number of samples tested, a range of quantitative values corresponding to normal and the different genotypes of α -thal syndromes can be defined, using a fluorescent spectrophotometer.

3). Detection of alpha gene from normal and α -thal syndrome patients by CCA.

PCR template and reaction conditions were the same as described in Example 1 above. When detection of PCR-CCA α gene in solution was used (directly visualization), amplified products were separated from free primers by ultrafiltration through Centricon C 100 microconcentrators after PCR. Colors were observed in the tubes under UV light. As shown in Figure 5A, both α_1 and α_2 genes from both normal and several α -thal patients have been successfully amplified. In normal subjects the existence of both α_1 and α_2 gene produced a color combination (yellow) from α_1 gene (green) and α_2 gene (red) (Figure 5. A1). In ($-\alpha/-\alpha$) patient only α_1 gene (green) was observed (Figure 5.A2), and in ($--/--$) patient, no color were detected (Figure 5.A3). We also confirmed the bands as specific amplified DNA fragments by visualizing with color photography from the TBE gels (Figure 5B). Two of each category subjects has been tested in this assay.

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- 20 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANTS: THE GOVERNMENT OF THE UNITED STATES OF AMERICA AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES; RODGERS, GRIFFIN, P.; TANG, DELIA C.
- (ii) TITLE OF INVENTION: A RAPID METHOD FOR DIAGNOSING THE VARIOUS FORMS OF α -THALASSEMIA.
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: MORGAN & FINNEGAN, L.L.P.
 - (B) STREET: 345 PARK AVENUE
 - (C) CITY: NEW YORK
 - (D) STATE: NEW YORK
 - (E) COUNTRY: USA
 - (F) ZIP: 10154
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: FLOPPY DISK
 - (B) COMPUTER: IBM PC COMPATIBLE
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: WORDPERFECT 5.1
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: TO BE ASSIGNED
 - (B) FILING DATE: 18 NOVEMBER 1997
 - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 60/031,880
 - (B) FILING DATE: 27-NOV-1996
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: FEILER, WILLIAM S.
 - (B) REGISTRATION NUMBER: 26,728
 - (C) REFERENCE/DOCKET NUMBER: 2026-4245PC
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (212) 758-4800
 - (B) TELEFAX: (212) 751-6849
 - (C) TELEX: 421792

(2) INFORMATION FOR SEQ ID NO:1:

- 21 -

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: UNKNOWN
 - (D) TOPOLOGY: UNKNOWN
- (ii) MOLECULE TYPE:
 - (A) DESCRIPTION: OLIGONUCLEOTIDE
- (ix) FEATURE:
 - (A) NAME/KEY: A PRIMER
 - (D) OTHER INFORMATION: 5' PRIMER COMMON TO BOTH ALPHA-1 AND ALPHA-2 GENES.
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGACCCTCTT CTCTGCACAG CTC

23

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: UNKNOWN
 - (D) TOPOLOGY: UNKNOWN
- (ii) MOLECULE TYPE:
 - (A) DESCRIPTION: OLIGONUCLEOTIDE
- (ix) FEATURE:
 - (A) NAME/KEY: B1 PRIMER
 - (D) OTHER INFORMATION: CORRESPONDS TO A REGION OF THE 3' UNTRANSLATED PORTION OF THE ALPHA-1 GENE.
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GAGGCCCAAG GGGCAAGAAG C

21

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23
 - (B) TYPE: NUCLEIC ACID
 - (C) STRANDEDNESS: UNKNOWN
 - (D) TOPOLOGY: UNKNOWN
- (ii) MOLECULE TYPE:
 - (A) DESCRIPTION: OLIGONUCLEOTIDE

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- (ix) FEATURE:
 (A) NAME/KEY: B2 PRIMER
 (D) OTHER INFORMATION: CORRESPONDS TO A
 REGION OF THE 3' UNTRANSLATED PORTION OF
 THE ALPHA-2 GENE.

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TTCCGGGACA GAGAGAACCC AGG

23

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WE CLAIM:

1. A nucleic acid composition comprising one or more of 5'-TGACCTCTTCTCTGCACAGCTC-3' (SEQ ID NO:1), 5'-GCGGCCCAAGGGGCAAGAAGC-3' (SEQ ID NO:2), and 5'-TTCCGGGACAGAGAGAACCCAGG-3' (SEQ ID NO:3).

2. A method of identifying multiply forms of α -thalassemia in a biological sample containing nucleic acids encoding hemoglobin comprising:

(a) hybridizing primers comprising a 5' common sense primer, a 3' α_1 - specific primer and a 3' α_2 primer to said nucleic acids encoding hemoglobin and an internal standard;

(b) extending the 3' ends of the primers forming an extension products;

(c) repeating steps (a) and (b); and

(d) detecting the products, wherein the presence of a form of α -thalassemia is indicated by a decrease in amount or an absence of one or more α hemoglobin extension products.

3. The method of claim 2 wherein the 3' α_1 -specific primer is 2 complementary to a 3' untranslated region of α_1 gene.

4. The method of claim 2 wherein the 3' α_2 -specific primer is complementary to a 3' untranslated region of α_2 gene.

5. The method of claim 2 wherein the 3' α_1 -specific primer comprises SEQ ID NO:2.

6. The method of claim 2 wherein the 3' α_2 -specific primer comprises SEQ ID NO:3.

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7. The method of claim 2 wherein the 5' common sense primer comprises SEQ ID NO:1.

8. The method of claim 2 wherein the biological sample comprises a dried blood sample.

9. The method of claim 2, wherein the extension products are separated prior to detection.

10. The method of claim 3 wherein separation is by electrophoresis.

11. The method of claim 2, wherein the primers are labeled.

12. The method of claim 2, wherein each of SEQ ID NOs:2 & 3 are labeled with different labels.

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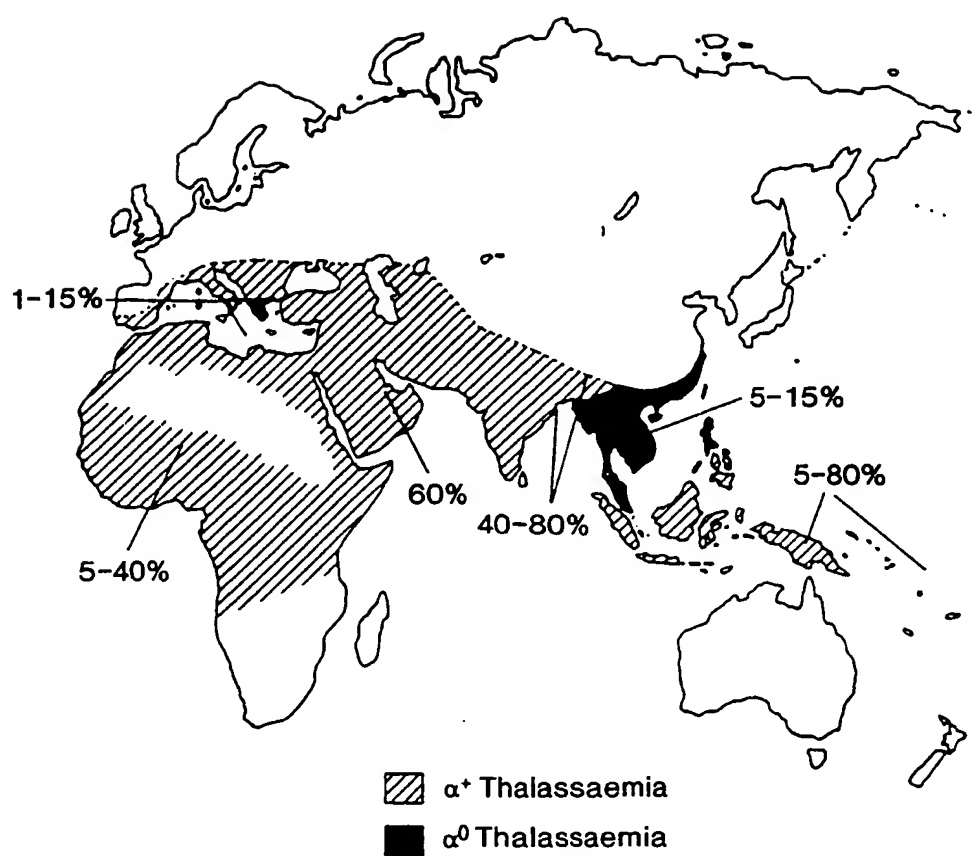
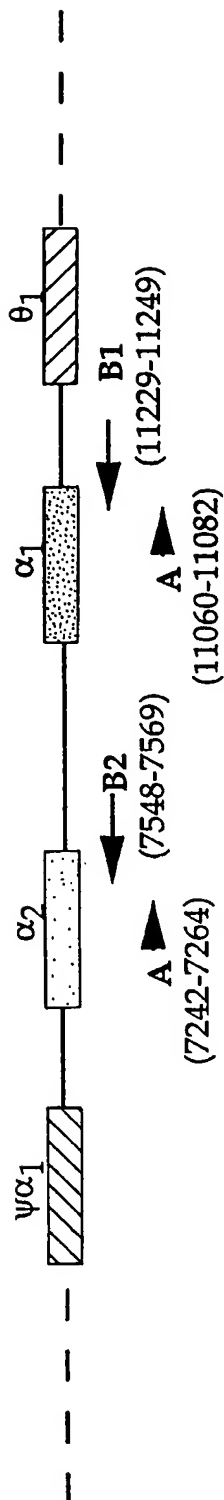


FIG. 1

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A = 5'-TGACCCTCTTCTCTGCACAGCTC-3'

B1 = 5'-GAGGCCCAAGGGCAAGAAGC-3'

B2 = 5'-TTCCGGGACAGAGAGAACCCAGG-3'

FIG. 2

CO-AMPLIFICATION OF α_1 - AND α_2 - GLOBIN GENES IN DNA FROM α - THALASSEMIAS

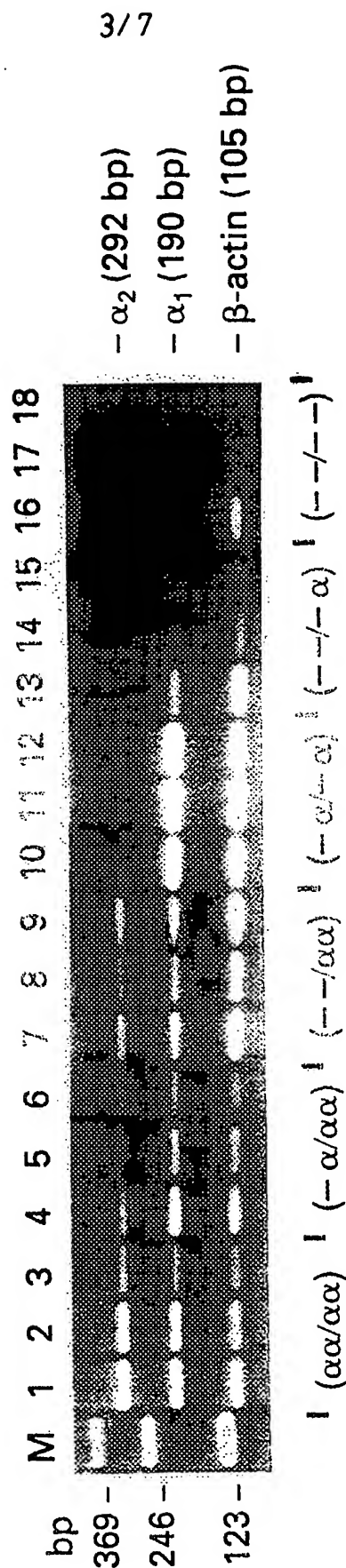


FIG. 3

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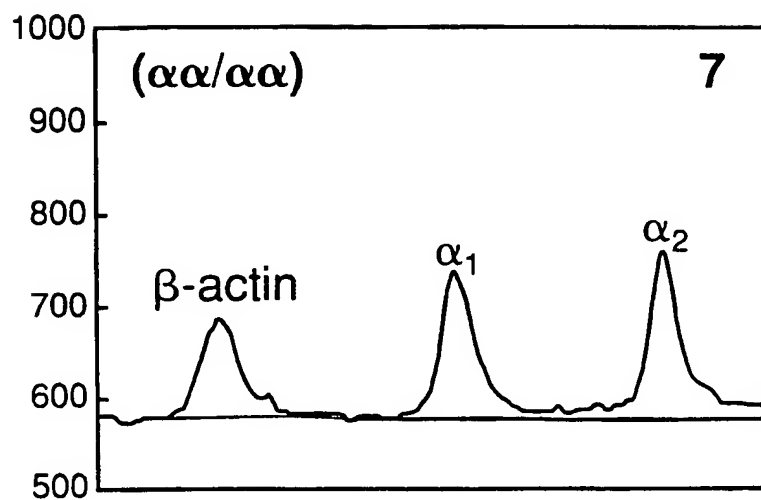


FIG. 4A

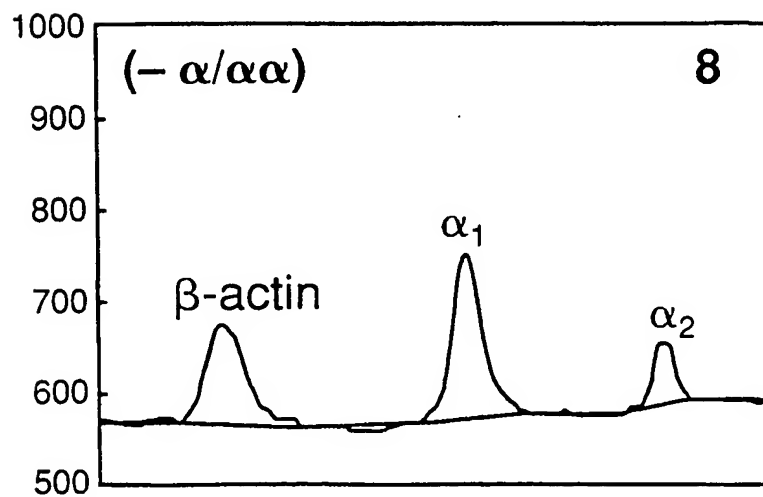


FIG. 4B

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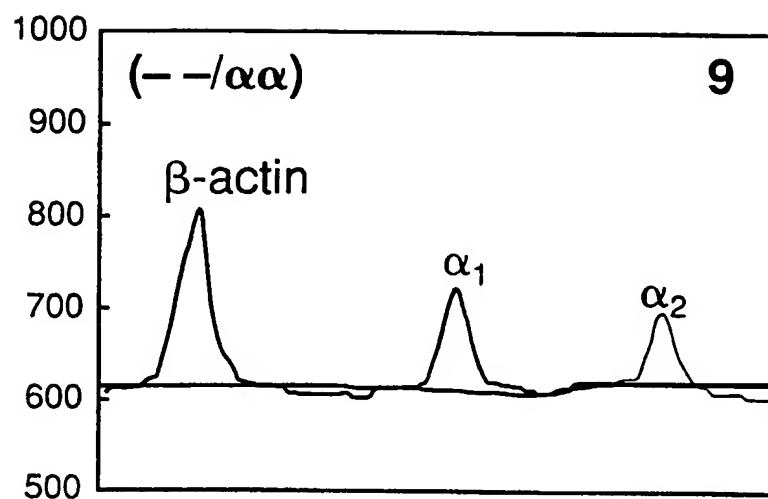


FIG. 4C

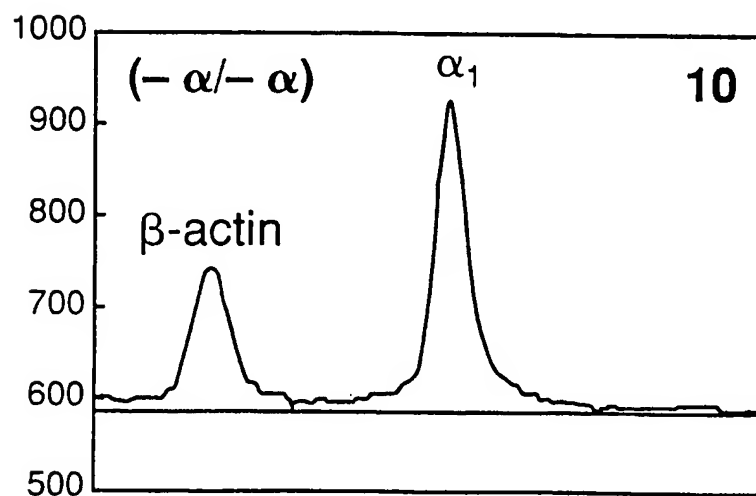


FIG. 4D

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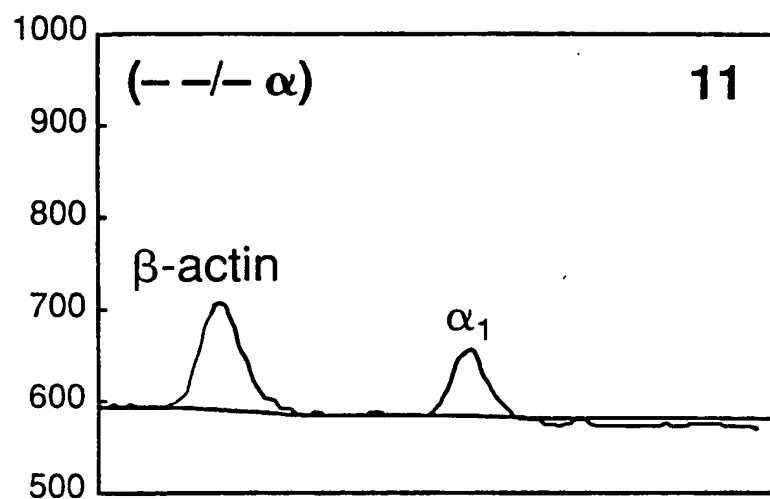


FIG. 4E

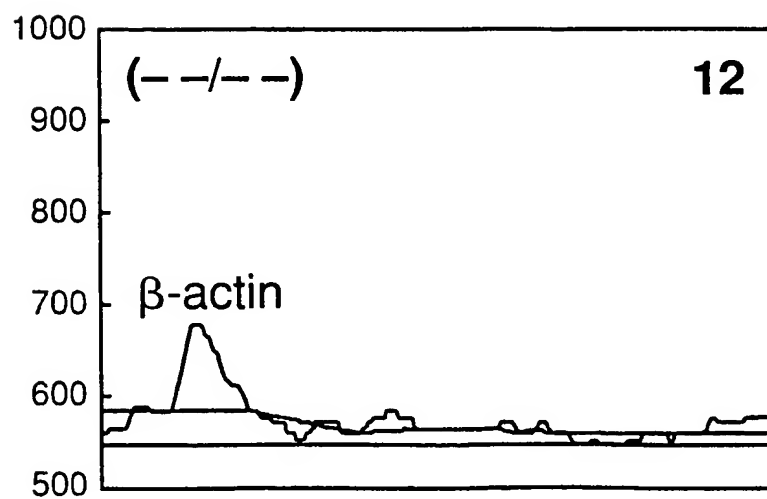


FIG. 4F

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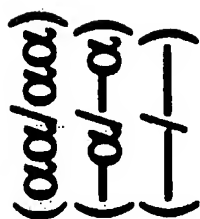
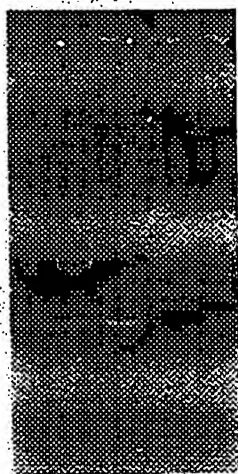


FIG. 5A



— α_2 (292bp)
— α_1 (190bp)

1 2 3



1 2 3

FIG. 5B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/21346

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BOWIE L. J. ET AL.,: "Detection of a-Thalassemias by multiplex polymerase chain reaction" CLIN. CHEM., vol. 40, no. 12, - 1994 pages 2260-2266, XP002060156 see results and discussion ---	2
X	WEN X-J. ET AL.,: "The nondeletional types of HB H disease in Guangxi" HEMOGLOBIN, vol. 16, no. 1&2, - 1992 pages 45-50, XP002060157 see the whole document --- -/--	2,9,10

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

25 March 1998

Date of mailing of the international search report

08/04/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Müller, F

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/21346

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LIU T-C. ET AL.,: "Molecular basis and hematological characterization of Hb H disease in southeast asia" AM. J. OF HEMATOLOGY, vol. 45, - 1994 pages 293-297, XP002060158 see materials and methods, discussion ----	2
A	ORKIN S.H. ET AL.,: "Mutation in an intervening sequence splice junction in man" PROC. NATL. ACAD. SCI. USA, vol. 78, no. 8, - August 1981 pages 5041-5045, XP002060159 see the whole document ----	1-12
A	BOWDEN D. K. ET AL.,: "A PCR-based strategy to detect the common servere determinants of a-thalassaemia" BRIT. J. HAEMATOLOGY, vol. 81, - 1992 pages 104-108, XP002060160 see the whole document ----	1-12
P,X	WO 97 16568 A (BOWIE LEMUEL J) 9 May 1997 see whole document, esp. claims -----	2

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No

PCT/US 97/21346

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9716568 A	09-05-97	NONE	